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PhiX-174 phage concentration in rainwater by skimmed-milk flocculation

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ABSTRACT

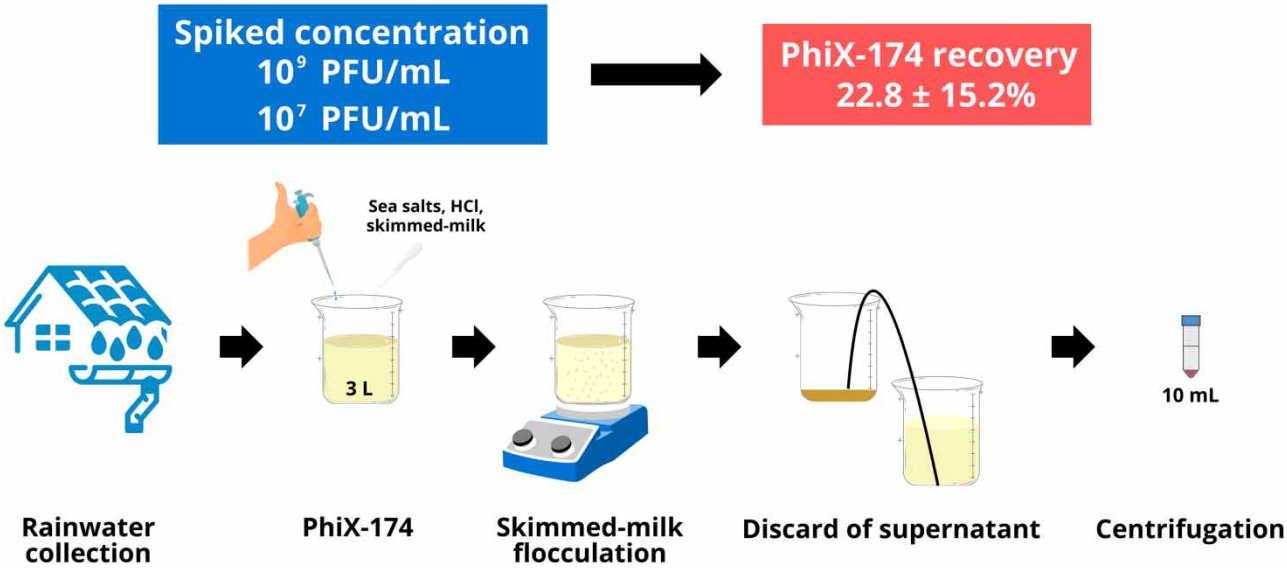
Rainwater, as a sustainable source of drinking water, is becoming increasingly important worldwide. Appropriate microbiological techniques are needed to ensure the acceptability of rainwater for potable uses. Bacteriophage has been widely used as a viral indicator in water studies, and its concentration is an important step when sampling several litres of water. Skimmed-milk flocculation (SMF) is a simple method for viral recovery, and its efficiency has already been assessed for some viruses. However, there are only a few records on bacteriophage recovery using SMF, and none with PhiX-174 coliphage, an important surrogate for human enteroviruses. Thus, this work aimed to evaluate the effectiveness of the SMF technique on the concentration of PhiX-174 coliphage in rainwater. 3 L of rainwater samples were artificially spiked with PhiX-174 and were concentrated to 10 mL volumes by SMF. The phage enumeration on the concentrated samples were evaluated by a standard double layer plaque assay. From the 6 samples tested, the average recovery was 22.8 $\pm$ 15.2%, ranging from 10.8 to 52.8%. This is the first time that SMF is applied for PhiX-174 coliphage recovery, and in rainwater. Therefore, SMF is a cost-effective method that can effectively be used to recover bacteriophage PhiX-174 in water samples.

Key words: bacteriophage, concentration, flocculation, rainwater, skimmed-milk, virus

HIGHLIGHTS

- PhiX-174 is a significant marker for water quality studies and can be found in rainwater.
- Viral concentration is an important step to work with large volumes of water samples.
- This is the first report of skimmed-milk flocculation applied to rainwater and PhiX-174.
- The average PhiX-174 recovery in rainwater with SMF was 22.8 $\pm$ 15.2%.

GRAPHICAL ABSTRACT



INTRODUCTION

It is estimated that at least two million people worldwide need to use water from sources contaminated with faeces, resulting in approximately 443,832 diarrhoea deaths annually (WHO 2024). Among waterborne pathogens, enteric viruses are the leading cause of outbreaks worldwide (Sinclair *et al.* 2009). Despite implementing current regulations on wastewater treatments, they are still widely disseminated in water and the environment through discharges of treated and untreated sewage to river catchments, coastal water and water used in food irrigation and shellfish production. Thus, human enteric viruses present an ongoing major public health hazard (Cantalupo *et al.* 2011). These viruses are amongst the smallest pathogens, making them extremely difficult to be removed by physical processes, such as filtration. Specific viruses may also be less sensitive to disinfection than enteric bacteria and some protozoan parasites (WHO 2018). Using human and animal viruses in laboratory testing is complicated, expensive, and therefore makes treatment comparisons more challenging.

The United States Environmental Protection Agency (USEPA) and the World Health Organization (WHO) have published guidelines for assessing the efficacy of devices used for water treatment, as well as disinfection methodologies for removing and/or inactivating viruses in water samples (USEPA 1987; WHO 2011). These methodologies recommend using two enteric viruses; however, the high costs involved in producing and testing human enteric viruses can be a limiting factor and a major impediment to testing in resource limited countries. These high costs can be substantially reduced if a bacteriophage is used as a surrogate for human enteric viruses (Gerba *et al.* 2015).

Bacteriophages are viruses that replicate only in bacterial cells and those that specifically infect coliform bacteria are known as coliphages (Lin & Ganesh 2013). These organisms are useful water quality indicators because they share several genetic, morphological, and behavioural similarities with enteric viruses (Pouillot *et al.* 2015) and are present in high concentrations in the sewage and faecal material of several mammals (McMinn *et al.* 2014). Resistance to environmental factors also makes them good candidates for this purpose (Jurzik *et al.* 2010). Based on this, the enumeration of coliphages has been widely accepted as a tool in water quality assessment (Grabow 2001). Among coliphages, PhiX-174 is a single-stranded DNA icosahedral virus, 27 nm in diameter with an isoelectric point of 6.62. It exhibits considerable resistance to halogen disinfectants such as iodine and chlorine (Gerba *et al.* 2015). These characteristics make it a potential indicator for water quality, especially those subjected to chemical disinfection.

Virus detection in polluted water requires concentration techniques and large volumes (100–1,000 L) of water are usually processed. These procedures are technically challenging and time consuming mainly because of the many steps involved, the need for several pH adjustments of the sample during processing and it is usually organism and/or matrix specific (Calgua *et al.* 2008). In response, a one-step protocol for the concentration of viruses was developed by Calgua *et al.* (2008) using costal water and later adapted to fresh water (Bofill-Mas *et al.* 2011; Calgua *et al.* 2013a). This method, based on a

skimmed-milk flocculation (SMF) was demonstrated to be useful, straightforward, and cost-effective and it was applied in routine water quality testing (Calgua *et al.* 2008; 2013a). Concentration of classic and emerging viruses such as human adenoviruses, polyomaviruses, and rotaviruses, by SMF has been previously conducted (Calgua *et al.* 2013a, 2013b; Gonzales-Gustavson *et al.* 2017; Garcia & Barardi 2019). However, interestingly, it is not commonly applied to bacteriophages, with only two reports available on bacteriophages MS2 and PP7 (Assis *et al.* 2017; Gonzales-Gustavson *et al.* 2017) and nothing related to PhiX-174, despite their significance as markers for human enteric viruses and as indicators in water quality guidelines (WHO 2018).

Accelerated population growth and the constant demand for urbanization associated with climate change have been largely responsible for the problem of drinking water scarcity for human consumption in some regions of the world. In light of this, domestic rainwater harvesting has been used as a supplementary or even sole source of drinking water in some water-deficient areas (Waso *et al.* 2020). Due to the accumulation of pollutants from the air or rain-collecting surfaces, rainwater usually presents poor quality, which is reflected by the presence of microbial pathogens (Ahmed *et al.* 2011). Considering the increase in demand and limited knowledge about the physical-chemical and microbiological characteristics of rainwater, it is important to guarantee the security of this source for human consumption, including the detection of viruses.

Although several studies have highlighted the SMF efficiency to concentrate viruses in different water matrices (Calgua *et al.* 2008, 2013a, b; Garcia *et al.* 2018), there is no information on the use of this technique in rainwater. Considering the evidence of viral presence in rainwater (Prado *et al.* 2022) and the feasibility of coliphages as a viral surrogate, the present study aimed to combine these two exponents to evaluate the effectiveness of the skimmed-milk flocculation technique on the concentration of the PhiX-174 coliphage in rainwater. This investigation seeks to evaluate the feasibility of this concentration technique as an alternative for monitoring rainwater for consumption purposes based on viral loads.

MATERIAL AND METHODS

Phage production

PhiX-174 (ATCC 13706-B1) and the host strain of *Escherichia coli* CN13 (ATCC 700609) were used for viral production. Both suspensions, originally lyophilized, were diluted in Tryptone Soy Broth (TSB) and phage buffer (1M Tris-HCL, MgSO4 and 2% Gelatin), respectively. A PhiX-174 stock suspension was serially diluted in 10-fold steps between 10^{-1} and 10^{-9}, in phosphate buffered saline (PBS) pH 7.4. Triplicate and 50 μL aliquots of each dilution were added together with 50 μL aliquots of the host *E. coli* in a tube containing 5 mL of sloppy agar (TSB and 0.5% bacteriological agar). This suspension was overlaid onto Petri dishes containing 15 mL of Tryptone Soya Agar (TSA) and incubated for 18–24 h at 37 °C. On the plates that exhibited complete bacterial lysis (>300 Plaque Forming Units (PFU)), 5 mL of phage buffer supplemented with 10% of glycerol was added and the plates were placed in an orbital shaker and gently agitated for 2 h at 18 rpm to release the bacteriophage into the liquid phase. The phage-containing liquid was transferred to 50 mL Falcon tubes and centrifuged at 2,000 x g for 15 min at 4 °C (Thermo Scientific® - Megafuge 8R). The supernatant was recovered and filtered through a 0.22 μm membrane filter coupled to a 20 mL syringe and stored at −80 °C.

Water sample and phage concentration

Rainwater was collected between November 2019 and February 2020 using fiber cement gutters installed on the roof of the Pilots Laboratory-USP, which, by vertical PVC conductors contained an outlet to eliminate the first rainwater. The rainwater was stored in a water tank until using it for viral concentration assays. Physical-chemical and bacteriological parameters of the rainwater were evaluated. To assess the efficiency of viral concentration, 3 L of rainwater was artificially contaminated with PhiX-174. Four samples were each spiked with 1.0×10^9 PFU and two samples with 1.0×10^7 PFU, achieving final concentrations of 3.3×10^8 PFU/L and 3.3×10^6 PFU/L, respectively. These inoculum concentrations were based on the WHO standard of $\geq 10^5$ PFU/mL for validating water treatment technologies (WHO 2018). This approach was used to determine whether the concentration technique could effectively handle samples with high levels of contamination without compromising quantification.

Skimmed-milk concentration was performed as described by Calgua *et al.* (2013a) and Garcia & Barardi (2019). First, the conductivity of the water samples was increased by adding 2.1 g of artificial sea salts (Sigma®) to achieve values >1,500 μS, and the pH was adjusted to 3.5 using 5M HCl. Then, a 1% (w/v) skimmed milk solution was prepared by dissolving 0.3 g of skimmed milk powder (Acumedia®) in 30 mL of distilled water with 1% (w/v) of artificial sea salts. The pH of skimmed milk

was adjusted to 3.5 using 1M HCl and the solution was added to the acidified water sample, achieving a final concentration of 0.01% of skimmed milk.

After stirring and a rest period of 8 h each, to allow sedimentation of the milk flocs, the supernatant was gently removed, and the bottom 400 mL were collected in centrifuge tubes. The sample was centrifuged at 3,260 *x* g, for 35 min at 4 °C (Thermo Scientific® - Megafuge 8R). The supernatant was removed, and the pellet was dissolved in 5 mL of 0.2 M phosphate-buffered at pH 7.5 (1:2, v/v of 0.2 M Na_2HPO_4 and 0.2 M NaH_2PO_4) and made up to a final volume of 10 mL. The sample was stored at 4 °C for one day to be used in the plaque assay enumeration method.

Plaque assay method

For viral enumeration, the double layer plaque assay was performed as described by (USEPA 2001). The concentrated sample from rainwater was first filtered through a 0.22 μm membrane, and then subjected to serial 10-fold dilutions in PBS between 10^{-1} and 10^{-9}. Following that, 50 μL of each dilution was added together with 50 μL of the host *E. coli* stock culture in a tube containing 5 mL of sloppy agar (TSB with 0.5% of bacteriological agar) and overlayed onto Petri dishes containing 15 mL of TSA. After agar solidification, the plates were incubated at 37 °C for 18–24 h and the number of PFU was counted. From each sample, two sets of dilutions were prepared, which were plated and counted independently. The following formula was used to calculate the PFU per mL of sample:

$$\text{Average no. of plaques} \times \frac{1,000}{\text{vol } (\mu\text{L}) \text{ of phage added}} \times \text{reciprocal dilution}$$

The percentage of viral recovery was calculated using the following formula:

$$\frac{\text{PFU per mL from the concentrated rainwater sample} \times 10}{\text{Concentration of phage added to the 3-L rainwater sample}} \times 100$$

The Kruskal-Wallis test was performed using the PAST 3.25 software to compare the recovery rate between the samples with different phage concentrations.

RESULTS AND DISCUSSION

Rainwater quality

Physical-chemical and bacteriological parameters of the rainwater collected are presented in Table 1. The rainwater matrix showed a low input of suspended particles, resulting in a turbidity close to 1 NTU, but a considerable concentration of dissolved particles (true color of 19.87 HU) and heavy microbiological contamination, with *E. coli* at 10^4 and total coliforms at 10^5. These results demonstrate the intrinsic complexity of a natural rainwater matrix, which, despite appearing clear, contains significant colour and microbiological organic load. Therefore, comprehensive studies are encouraged, particularly those that include analyses of other physicochemical and microbiological parameters such as dissolved organic carbon, scanning absorbance measurements, and the quantification of a range of viruses and bacteriophages.

Table 1 | Physical-chemical parameters of the rainwater samples used for phiX-174 recovery test

Parameter	Units	Value (mean ± standard deviation)
pH	–	7.16 ± 0.26
Conductivity	μS cm^{-1}	56.06 ± 5.95
Turbidity	NTU	1.06 ± 0.92
Apparent colour	HU	21.93 ± 2.40
True colour	HU	19.87 ± 1.33
Escherichia coli	CFU /100 mL	1,800 ± 62
Total coliforms	CFU /100 mL	13,800 ± 95

PhiX-174 recovery by SMF

PhiX-174 concentration by skimmed milk flocculation in 3 L rainwater samples were performed with two different concentrations (3.3×10^8 and 3.3×10^6 PFU/L). The average viral recovery observed was $22.8 \pm 15.2\%$, with no significant distinction (*p-value* = 0.3476) between the samples with higher and lower virus concentrations (Table 2). The PhiX-174 recovery rate ranged between 10.8 and 52.8%, with the highest value observed when the virus was inoculated with 1.0×10^7 PFU.

Virus recovery by SMF was originally described for seawater samples (Calgua *et al.* 2008) and further applied in brackish water (Moresco *et al.* 2012). In order to concentrate viruses in freshwater, a protocol was developed by adjusting the water conductivity with artificial sea salts (Bofill-Mas *et al.* 2011). Since then, SMF has been used to recover viruses in different water samples, such as sewage, river, and tap water (Cantalupo *et al.* 2011; Calgua *et al.* 2013a, 2013b; Assis *et al.* 2017; Garcia *et al.* 2018). This is the first report of virus concentration in a rainwater sample.

Virus recovery rate by SMF has shown variable results in previous studies, due to variations between water matrices and volumes concentrated. In seawater, Calgua *et al.* (2008) observed 42–49% of recovery, while Moresco *et al.* (2012) and Garcia *et al.* (2015) obtained 10% and 31–33%, respectively. In sewage samples, recovery rates ranged between 30–95% (Calgua *et al.* 2013b) and 25–84% (Assis *et al.* 2017). When applied in freshwater, virus recovery in river samples was around 50% (Calgua *et al.* 2013a) and in tap water it ranged between 14–66% (Gonzales-Gustavson *et al.* 2017) and 26–53% (Garcia & Barardi 2019). The average viral recovery rate for rainwater was 22.8%, ranging from 10.8 to 52.8%. This relative low recovery average could be due to the lower ionic strength in rainwater compared to other sources, even though the conductivity was adjusted with artificial sea salts.

However, it has been noted that the viral quantification method applied can contribute to a variation in recovery rates among the different studies. Higher values of recovery were usually observed when viruses were quantified by qPCR than by infectivity assays (Gonzales-Gustavson *et al.* 2017). While the recovery rate by qPCR from human adenovirus (HAdV) was observed multiple times above 50% (Calgua *et al.* 2008, 2013a; Assis *et al.* 2017; Gonzales-Gustavson *et al.* 2017), infectivity assays showed rates between 31 and 58% (Garcia *et al.* 2015; Gonzales-Gustavson *et al.* 2017; Garcia & Barardi 2019). Although the plaque assay method is the simplest and more accurate to assess virus infectivity, a comparison of the recovery rate by a molecular method, such as qPCR, could be performed in further studies.

The difference on the target organism may also influence SMF efficiency, probably due to a difference in the isoelectric point and their capacity to aggregate to the milk flocs. Human viruses, such as HAdV, norovirus and poliomavirus, showed recovery rates higher than 50% (Calgua *et al.* 2008, 2013a; Gonzales-Gustavson *et al.* 2017), while for zoonotic (rotavirus) and animal (bovine viral diarrhoea virus) viruses, the recovery was below 30%. Garcia & Barardi (2019) also observed a variation in the recovery rate for different viruses, which was 53% for HAdV and 26% for murine norovirus. However, only limited studies have evaluated the efficiency of SMF on bacteriophage concentration.

Gonzales-Gustavson *et al.* (2017) observed a MS2 average recovery in tap water of 23.9% and 11.9% by qPCR and plaque assay, respectively. PP7 was used as an indicator in the sewage sample, showing a recovery rate between 12 and 16% by qPCR (Assis *et al.* 2017). This is the first report showing PhiX-174 concentration in water by SMF, which with an observed average recovery of 22.8%, places it within the reported range of bacteriophage recovery determined using the plaque assay method.

Table 2 | PhiX-174 recovery data by skimmed milk flocculation in 3 L rainwater samples artificially contaminated with two different phage concentrations

Sample	Phage inoculated (PFU)	Phage recovery (PFU/mL)	Phage recovery (%)
1	1.0×10^9	1.40×10^7	14.0
2	1.0×10^9	2.00×10^7	20.0
3	1.0×10^9	1.08×10^7	10.8
4	1.0×10^9	2.00×10^7	20.0
5	1.0×10^7	5.70×10^5	52.8
6	1.0×10^7	2.06×10^5	19.1
Average recovery			22.8

The initial concentration of virus in rainwater seems not to interfere with the recovery rate. In fact, the highest recovery rate was observed with the lower quantity of added virus. We recognize that lower concentrations of PhiX-174 in rainwater should be tested in the future, however previous studies have demonstrated consistent results with different initial concentrations. Calgua *et al.* (2008) added approximately 5.0×10^3 genome copies/L of HAdV and observed around 50% of recovery. Garcia & Barardi (2019), spiked HAdV with 1.0×10^6 focus forming unit/L and showed 53% of recovery. Consistent data were also observed by Calgua *et al.* (2013a), which obtained similar virus recovery rates, although the initial concentration varied between 10^7 and 10^{11} genome copies/L.

CONCLUSIONS

In addition to somatic coliphage monitoring, viral removal, and disinfection studies in pilot or real scale may require a concentration method to obtain significant data. Thus, SMF is a cost-effective method that can effectively be used to recover bacteriophage PhiX-174 in water samples, including rainwater. This is the first report of SMF in rainwater samples using PhiX-174 as the viral indicator and the average recovery was $22.8 \pm 15.2\%$. Given these findings, further research is recommended to evaluate this concentration technique for other viruses and bacteriophages in both controlled and natural samples.

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DATA AVAILABILITY STATEMENT

All relevant data are included in the paper or its Supplementary Information.

CONFLICT OF INTEREST

The authors declare there is no conflict.

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